Genetic Engineering of Streptavidin-Binding Peptide Tagged Single-Chain Variable Fragment Antibody to Venezuelan Equine Encephalitis Virus

This Application claims the benefit of U.S. Provisional Application No. 60/448,902, filed on February 24, 2003, the entire content of which is incorporated by reference in this application.

FIELD OF THE INVENTION

This invention relates to the construction of a recombinant gene encoding a single-chain variable fragment antibody cloned into an expression vector and fused with a streptavidin-binding peptide sequence to produce a fusion protein. The resultant fusion protein can be used as reagent for immunoassay of Venezuelan equine encephalitis virus when detected by horseradish peroxidase-conjugated streptavidin. This invention is related to U.S. Provisional Patent Application No. 60/361,698 filed by Fulton et al., the same inventors and assignee, which is herein incorporated by reference.

BACKGROUND OF THE INVENTION

List of Prior Art Literatures

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Venezuelan equine encephalitis virus (VEE), belonging to alphavirus genus of the family *Togaviridae*, is an important pathogen of epidemics in humans and of epizootics in some animals (Johnston et al., 1996). VEE causes a spectrum of human diseases ranging from inapparent infection to acute encephalitis (Franck et al; 1970; Johnson et al., 1968). Since the VEE genome is composed of positive sense RNA, its nucleic acid is infectious independent of the complete viral particle (Johnston et al., 1996). Furthermore, VEE is highly infectious by aerosol inhalation in humans (Johnston et al., 1996). Thus, VEE is a potential biological warfare and bioterrorist agent of concern. Therefore, simple, stable, and efficient immunoassays are required for rapid identification of VEE in environmental or clinical samples in order that immediate therapeutic and preventive counter measures can be taken to limit the epidemic spread of VEE infection.

The present inventors have previously cloned and characterized several single-chain variable fragment antibodies (scFv Abs) against VEE (Alvi et al., 1999; Alvi et al., 2002; Alvi et al., 2003). Among them, mA116 scFv Ab was well characterized, showing sensitivity and specificity in recognition of VEE by immunoassay (Alvi et al., 2003). In order to further explore the potentiality of mA116 scFv Ab as an immunodiagnostic reagent for detecting VEE, the present inventors successfully fused a streptavidin-binding peptide (SBP) to mA116 scFv Ab by DNA recombinant technique. This confers a streptavidin-binding function on the mA116 scFv Ab and therefore obviates the need for conventional chemical biotinylation. Chemical biotinylation is commonly associated with impairment of the antigen-binding site of the Ab and it is hence desirable to use the recombinant SBP tagged mA116 scFv of the present invention as reagent to develop a simple, stable and efficient immunoassay for VEE.

SUMMARY OF THE INVENTION

It is an object of the present invention to teach a method for constructing a streptavidin-binding peptide (SBP) to the sequence for mA116 scFv Ab to VEE. According to one aspect of the present invention, it provides a method for constructing a recombinant gene encoding a single-chain variable fragment antibody cloned into an expression vector and fused with a SBP gene sequence to produce a fusion protein, comprising: (a) encoding anti-VEE scFv Ab gene to a recombinant plasmid and inserting a SBP gene and a 6His tag downstream to develop a SBP tagged scFv Ab construct; (b) amplifying the resultant scFv/SBP/6His by polymerase chain reaction; (c) inserting the amplified PCR products into cloning vector to produce a SBP-plasmid; (d) constructing

said SBP-plasmid with promoter to produce a SBP tagged scFv Ab; and (e) expressing said SBP tagged scFv Ab in *E. coli* cells as inclusion bodies and purifying the expressed SBP tagged scFv Ab by immobilized metal affinity chromatography.

It is another object of the invention to demonstrate that the recombinant fusion protein retains antigen-binding affinity to VEE and possesses streptavidin-binding function. Hence, the genetically recombinant SBP tagged mA116 scFv Ab can be used as an excellent reagent for detecting VEE by means of immunoassay. According to another aspect of the present invention, it provides a method for using the SBP tagged recombinant scFv Ab fusion protein of claim 4 for detecting VEE, comprising: (a) reacting the SBP tagged scFv Ab with a sample containing VEE for observing antigen-binding activity; and (b) analyzing the reactant by enzyme-linked immunosorbent assay (ELISA).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing the construction of pCRT7mA116SBP. Step 1 - A single double-stranded oligonucleotide encoding SBP and 6His flanked by Not I and Sal I sticky ends was ligated into pPICZαBmA116 digested with NotI/Sal I. Step 2 - scFv/SBP/6His tag sequence was amplified from pPICZαBmA116SBP vector by PCR. Step 3 - PCR product was ligated into pCRT7 TA cloning vector as described under "Materials and Methods".

Figure 2 shows the nucleotide and deduced amino acid sequences (SEQ ID NOs. 1 and 2 respectively) of SBP tagged mA116 Ab. MA116 scFv followed by SBP and 6His tags.

Figure 3 shows SDS-PAGE analysis of samples from the purification of the SBP tagged mA116 scFv Ab. Samples were resolved on 10 % polyacrylamide gel and stained with Coomassie blue. Lane 1 - molecular weight markers; Lane 2 - bacterial lysate; Lane 3 - solubilized protein fraction; Lane 4 - column flow through fraction; Lane 5 - purified fraction.

Figures 4A & 4B show Western blotting analysis of samples from the purification of the SBP tagged mA116 scFv Ab. Samples were resolved by SDS-PAGE, transferred to Immunobilon-P membranes, and probed with: Figure 4A: HRP-conjugated Ni-NTA. Figure 4B: HRP-conjugated streptavidin. Lane 1- bacterial lysate; Lane 2- solubilized protein fraction; Lane 3- column flow through fraction; Lane 4- purified fraction.

Figures 5A & 5B are graphs showing VEE antigen binding assay by ELISA. Figure 5A: Various concentrations of Abs were added to 96-well plate coated with 10 μg/ml of VEE; Figure 5B: 10 μg/ml of Abs were added to a 96-well plate coated with various concentrations of VEE. Binding was detected with HRP-conjugated streptavidin or HRP-conjugated anti-mouse Ig followed by ABTS solution. Each point represents the mean ± the standard error of the mean (SEM) of the four determinations.

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

Construction of pCRT7mA116SBP

The pPICZαBmA116 recombinant plasmid, containing anti-VEE mA116 scFv Ab gene, arranged in variable heavy (VH)-variable light (VL) chain orientation via $(Gly_4Ser)_3$ linker, was constructed previously. In order to introduce a SBP sequence,

PCHPQFPRCYA (SEQ ID NO. 3) (Lue et at., 1998) followed by a 6His tag at the C-terminus of mA116 scFv Ab, two complementary oligonucleotides corresponding to the SBP sequence and 6His tag with flanking sequences for restriction enzymes Not I and Sal I, were synthesized and purified by Life Technologies (Burlington, ON). The sequences were as follows: sense, 5'-ggccgcCCATTCTGGTGGTGGTGGCCCATGCCATCCGC AGTTCCCACGATGTTATGCGGGTGGTGGCGGTTCTCATCATCATCATCATCAT TGAg-3' (SEQ ID NO. 4); anti-sense, tcgacTCAATGATGATGATGATGATGAGAAC CGCCACCACCCGCATAACATCGTGGGAACTGCGGATGGCATGGGCCACCACC ACCAGAATGGgc-3' (SEQ ID NO. 5). The two oligonucleotides were heated to denature, and then annealed to a single double-stranded oligonucleotide by slow cooling to room temperature. The annealed dimer possessed a Not I sticky end on one side and Sal I on the other side, and was ligated to pPICZαBmA116 that had been cut with Not I and Sal I. The resulting plasmid was named pPICZαBmA116SBP.

To obtain high expression of the recombinant fusion protein, the PCR method was introduced to amplify the mA116 scFv/SBP/6His sequence in pPICZαBmA116SBP vector and the PCR product was subcloned into a T7 RNA polymerase-regulated expression vector. Two primers were synthesized on an Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, CA). The sequence of the forward primer was 5'-ATGGCTAAAGAAGAAGGGGTATC-3' (SEQ ID NO. 6) and the reverse was 5'-TCATGTCTAAGGCTACAAACTCAA-3' (SEQ ID NO. 7). PCR reaction in a 50µl volume consisted typically of 200 µmol each dNTP, 0.6 µM primers, 0.1 µg template, and 1.25 unit of HotStarTaq™ DNA polymerase in buffer supplied by the manufacturer (Qiagen, Mississauga, ON). Initial activation (95°C for 15 min) was carried out followed

by cycling (94 °C for 1 min, 61 °C for 1 min, and 72 °C for 2 min), repeated 30 times, on a Peltier Thermal Cycler (DNA Engine PTC-200; MJ Research, Watertown, MA). After gel-purification, the PCR fragment was cloned into the pCRT7 vector by use of a pCRT7 TA cloning expression kit in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). The recombinant plasmid, named pCRT7mA116SBP, contained the correct orientation of the insert, mA116 scFv/SBP/6His tag, as confirmed by restriction digestion fragment mapping and DNA sequencing.

Expression, purification and refolding of the SBP tagged mA116 scFv Ab

Expression, purification and refolding of the SBP tagged mA116 scFv Ab were performed using minor modifications of previously described methodologies (Long et al., 2000). In brief, an overnight culture of *E. coli* BL-21 (DE3) pLys cells that had been transformed with pCRT7mA116SBP vector, was diluted 1:50 with LB-medium containing 100 μg/ml ampicillin and incubated with shaking at 37 °C to OD_{600} of 0.5. The promoter was then induced for 3 hr by isopropyl β-D-thiogalactoside (IPTG). The cell pellet was resuspended in 5 mM borate sodium, pH 9.3 and 4 M urea, and cell lysate was prepared by sonication (three cycles of 10 sec; amplitude 10 μm; 15 sec cooling on ice), using a MSE Soniprep 150-probe sonicator (Wolf Laboratories, Pocklington, UK). The sonicates were centrifuged (13,000 g for 10 min) and pellets were resuspended in 5 mM borate sodium, pH 9.3, 8 mM urea, and 100 mM sodium chloride (solubilizing agent). Purification of the recombinant protein was performed on Talon™ metal affinity resin (Clontech, Palo Alto, CA). A solution of 5 mM borate sodium, pH 9.3, 8 M urea, and 100 mM sodium chloride was used as wash buffer. Bound fractions were eluted with 100 mM imidazole and then 1 M arginine (final concentration) was added as cosolvent,

to encourage the correct folding of the protein molecules. The recombinant protein was refolded by removal of 8 M urea, by dialyzing against 5 mM borate sodium, pH 9.3, and 1 M arginine; the cosolvent was then removed by dialyzing against 5 mM borate sodium, pH 9.3. The purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Mississauga, ON) staining after samples had been concentrated in dialysis bags on a bed of polyethylene glycol compound, molecular weight (MW) 15,000-20,000 (Sigma, Oakville, ON).

SDS-PAGE and Western blot analysis

Proteins were separated by 10% SDS-PAGE gels by use of a Mini-PROTEAN II apparatus (Bio-Rad Laboratories). The bands were visualized by Coomassie blue staining. The molecular weights of the samples were estimated by comparison to the relative mobility values of standards of known molecular weights.

Gels were immunoblotted to Immunobilon-P membranes (Millipore Corp, Bedford, MA) using a western blot semi-dry transfer apparatus (Bio-Rad Laboratories) with Towbin buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol). Blots were blocked with blocking buffer (2% bovine serum albumin in phosphate-buffered saline (PBS)). Blots were washed three times for 5 min with PBS containing 0.1% tween-20 (PBST) and then incubated directly with a 1:1000 dilution of HRP-conjugated streptavidin (Sigma) or a 1:2000 dilution of HRP-conjugated Ni-nitrilotriacetic acid (NTA) (Qiagen) at room temperature for 1 hr. After three washes for 5 min with PBST, and two washes for 2 min with deionized water, the specific binding

was detected by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Baie d'Urfe, QC).

Enzyme-linked immunosorbent assay (ELISA)

The antigen-binding activity of the purified SBP tagged mA116 scFv Ab to VEE antigen was determined by an ELISA. Nunc maxisorp™ flat-bottomed 96-well plates (Life Technologies) were coated overnight at 4 °C with whole VEE (strain TC-83) at a fixed concentration of 10 μg/ml, or various concentrations of 0.2-60 μg/ml, in carbonate bicarbonate buffer, pH 9.6, containing 0.02% sodium azide. The plates were washed five times with PBST and then blocked twice in 2% bovine serum albumin for 1 hr at 37 °C. After five washes with PBST, plates were incubated for 1 hr at 37°C with various concentrations of 0.6-50 μg/ml, or a fixed concentration of 10 μg/ml of the SBP tagged mA116 scFv Ab or its parental monoclonal antibody (MAb) 1A4A1, diluted in PBST. Following five washes with PBST, plates were incubated for 1 hr at 37°C with 1:1000 dilution of HRP-conjugated streptavidin in PBST for the SBP tagged mA116 scFv Ab and 1:2000 dilution of HRP-conjugated goat anti-mouse Ig for 1A4A1 MAb. Finally, the plates were washed five times with PBST and developed for 30 min at room temperature with a substrate consisting of 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxidate (Kirkegaard and Perry Laboratories, Gathersburg, MD). The reactions were read at an absorbance of 405 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA).

Results

Construction, expression and purification

The pPICZαBmA116 recombinant plasmid, encoding anti-VEE mA116 scFv Ab gene was used as a source material to create the SBP tagged mA116 scFv Ab construct. After a synthetic double-stranded oligonucleotide encoding a SBP and 6His tag was inserted downstream to the pPICZαBmA116, mA116 scFv/SBP/6His was amplified using PCR method with appropriate primers. The PCR product was inserted into pCRT7 TA cloning vector. The resulting plasmid, designated pCRT7mA116SBP, contained the mA116 scFv Ab gene, followed by the SBP sequence under the control of T7 promoter (Figure 1). In addition, there was a 6His tag located downstream of the SBP for immobilized metal affinity chromatography (IMAC) purification. The nucleotide and deduced amino acid sequences are showed in Figure 2. The encoded whole recombinant fusion protein was 296 residues with a predicted molecular weight of 31.3 kDa.

The SBP tagged mA116 scFv Ab was expressed in *E.coli* BL-21 cells as inclusion bodies and purified by IMAC. SDS-PAGE demonstrated that there was a relatively small amount of protein in the bacterial lysate of molecular weight ~32 kDa corresponding to the predicted size (31.3 kDa) of the SBP tagged mA116 scFv Ab, due to the presence of large amounts of contaminating proteins (Figure 3, Lane 2). However, after centrifugation of the lysate, and dissolution of the pellet in solubilizing agent, many of the bacterial host proteins were removed from the lysate, making the ~32 kDa band more visible (Figure 3, Lane 3). The solubilized protein fraction was incubated with metal affinity resin and loaded to an empty column. After thoroughly washing with wash buffer, the bound fractions were eluted by 100 mM imidazole. Only one band of ~32 kDa was observed in the purified fraction (Figure 3, lane 5). In this purification protocol, the expressed protein could be purified to over 90%.

Biochemical characterization

To confirm the integrity of the expressed SBP tagged mA116 scFv Ab, a series of Western blotting experiments was performed, in which the 32-kDa protein was detected by both HRP-conjugated streptavidin and HRP-conjugated Ni-NTA (Figure 4). With HRP-conjugated Ni-NTA, a 32 kDa band was observed in all purification fractions (Figure 4A, Lanes 2-4). With HRP-conjugated streptavidin, bands were visible at 32 kDa in all fractions (Figure 4B, Lanes 2-4). In addition, bands were observed at 20kDa in the bacterial lysate, solubilized protein fraction, and column flow through fraction (Figure 4B, Lanes 1-3). However, after purification, only the 32 kDa band was present in the purified fraction (Figure 4B, Lane 4).

Binding properties to VEE antigen

The immunoreactivity of the SBP tagged mA116 scFv Ab to VEE antigen was examined by ELISA. When the plates were coated with a fixed concentration of VEE (10μg/ml), the SBP tagged mA116 scFv Ab bound to VEE in a dose-dependent manner, similar to the binding to VEE of its parental 1A4A1 MAb (Figure 5A). An additional ELISA was performed in which a concentration gradient of VEE was titrated against a fixed concentration of Abs (10 μg/ml). A similar dose-response relationship was observed (Figure 5B).

Discussion

Since the introduction of MAb technology, a number of types of immunodiagnostic assays have been developed based on specific binding between antigens and their corresponding MAbs (Nakamura, 1983). However, the disadvantages

of MAbs as immunodiagnostic reagents are numerous. The cost and time required for growth and maintenance of hybridoma cell lines, and production and purification of MAbs, coupled with the potential for occurrence of genetic mutation during repeated cycles of cell growth, makes routine production of MAbs from hybridoma cell lines difficult, expensive, and time consuming. ScFv Abs are comprised of immunoglobulin VH and VL chains, covalently connected by a peptide linker (Huston et al., 1988). These small proteins generally retain the specificity and affinity for antigen similar to their parental MAb and possibly bind to poorly accessible epitopes more efficiently due to their small size (Marin et al., 1995; Bruyns et al., 1996). The attractiveness of scFv Abs is that they can be produced economically in bacteria and manipulated via genetic engineering, for example to form fusion proteins with additional functions (George et al., 1995; Boleti et al., 1995; Wels et al., 1992).

The streptavidin-biotin system has one of the highest affinities (10^{-15} M) among receptor-ligand interactions (Green, 1963). The strong interaction between streptavidin and biotin has been applied in many immunoassays (Guesdon et al., 1979; Hsu et al., 1981). However, chemical biotinylation of Ab is time-consuming and, as most of the biotin binds to amino groups of the protein, the degree of labeling can differ from batch to batch. Furthermore, the possibility exists that the biological activity of the Ab may be affected by the labeling procedure (Mirables et al., 1991). With advent of recombinant DNA technology, it is possible to fuse a short peptide to the target protein through gene fusion technique. SBPs, constituting around 10 amino acids have been selected from random peptide libraries (Devlin et al., 1990; Osterguard et al., 1995; Gissel et al., 1995). Some of them have been well characterized (Schmidt et al., 1996; Skewa et al., 1999).

SBPs have successfully been fused to scFv Abs to antigen CA125, *Bacillus cereus* spores, and scorpion toxin for use in immunoassay (Luo et al., 1998 Kao et al., 1998 Aubrey et al., 2001).

The present inventors genetically incorporated a SBP sequence in mA116 scFv Ab gene in order to biotinylate mA116 scFv Ab. DNA sequencing confirmed that DNA cloning was successful. The SBP tagged mA116 scFv Ab was expressed in *E. coli* to high levels in the form of insoluble inclusion bodies. The insoluble recombinant fusion protein was solubilized by denaturing agent, 8M urea. Inclusion of 6His tag allowed the solubilized recombinant fusion protein to be purified via IMAC. In this way, greater than 90% purity of the SBP tagged recombinant mA116 scFv Ab could be obtained. After purification, arginine was introduced to the recombinant protein solution to direct correct refolding.

The streptavidin-binding peptide confers reversible binding activity toward the streptavidin. Therefore, it can be employed for the one step purification of a corresponding fusion protein via streptavidin affinity chromatography (Schmidt et al., 1994; Zwicker et al., 1999). However, in the present invention, purification of the recombinant SBP tagged mA116 scFv Ab using streptavidin affinity column chromatography yielded only small amounts of product containing relatively large amounts of host proteins (data not shown). In fact, a large amount of streptavidin-binding protein of around 20 kDa showed up in the bacterial lysate on Western blot analysis of bacterial lysate (Figure 4B). This may be attributed to whole cell extracts of *E.coli* containing biotinylated proteins, such as biotin carboxyl carrier protein (22.5 kDa) that binds strongly to streptavidin (Sutton et al., 1977). Accordingly, a 6His tag was

introduced into the gene construct in order that IMAC could be used to purify the Ab of the present invention.

The results of Western blot analysis confirmed that the refolded recombinant fusion protein was intact, with a molecular weight of ~32kDa. The *in vitro* binding characteristics of the SBP tagged mA116 scFv Ab to VEE antigen were assayed by ELISA. The recombinant fusion protein exhibited strong binding activity to VEE, indicating that the SBP did not interfere with the conformation of antigen-binding site or the bioactivity of mA116 scFv Ab. The parental 1A4A1 MAb showed similar binding activity to VEE. However, a direct comparison of the binding affinities between both Abs was not possible by ELISA due to the use of different HRP-conjugates.

In summary, a SBP sequence was introduced downstream to the sequence for mA116 scFv Ab to VEE. The fusion protein was expressed and purified. Fusion to the SBP did not affect the ability of mA116 scFv Ab to recognize VEE antigen with an affinity similar to that observed for the parental MAb. Similarly, the streptavidin-binding property of the fusion protein was not impaired. Western blot and ELISA results suggest that SBP tagged mA116 scFv Ab could be used for simple, stable, and efficient detection of VEE when used in conjunction with HRP-conjugated streptavidin. This approach eliminates the need for chemical biotinylation of Abs with resultant possible impairment of the antigen-binding site of the Ab.

It is to be understood that the embodiments and variations shown and described herein are merely illustrative of the principles of this invention and that various modifications may be implemented by those skilled in the art without departing from the scope and spirit of the invention.

In addition, the List of Prior Art Literatures referred to in the Background of the Invention section is incorporated by reference herein.